

1 METHOD FOR MODIFICATION OF NMDA RECEPTORS THROUGH INHIBITION
2 OF SRC
3

4 FIELD OF THE INVENTION

5 The instant invention relates generally to protein-
6 protein interactions that regulate intra and intercellular
7 communication; particularly to methods for modification of
8 protein-protein interactions; and most particularly to a
9 method for modifying the activity of NMDA (N-methyl-D-
10 aspartate) receptors located in cells by inhibition of the
11 interaction of the unique domain of the tyrosine kinase Src
12 enzyme with proteins of the NMDAR complex.
13

14 BACKGROUND OF THE INVENTION

15 Excitatory transmission at central synapses is primarily
16 mediated by the amino acid glutamate acting through
17 postsynaptic ionotropic receptors (Dingledine et al.
18 Pharmacological Review 51:7-61 1999). The N-methyl-D-
19 aspartate receptor (NMDAR) is one such type of ionotropic
20 glutamate receptor (Dingledine et al. Pharmacological Review
21 51:7-61 1999). NMDARs are multiprotein complexes located at
22 excitatory synapses within the postsynaptic density (PSD)
23 comprised of the core channel subunits together with
24 associated scaffolding and regulatory proteins that control

1 receptor localization, ionic flux through the receptor and
2 downstream signaling events (Scannevin et al. Nature Reviews
3 Neuroscience 1:133-141 2000; Sheng et al. Annual Review of
4 Physiology 62:755-778 2000). NMDAR's are crucial for central
5 nervous system (CNS) development, neuroplasticity and
6 pathophysiology (Dingledine et al. Pharmacological Review
7 51:7-61 1999; Sheng et al. Science 298:776-780 2002).
8 Multiple factors regulate NMDAR function, including dynamic
9 cycling of protein phosphorylation and dephosphorylation at
10 serine/threonine or tyrosine residues (Wang et al. Nature
11 369:233-235 1994; Smart Current Opinion in Neurobiology
12 7:358-367 1997). The Src protein is one such factor that
13 modulates the activity of the NMDARs (Yu et al. Science
14 275:674-678 1997; Lu et al. Science 279:1363-1368 1998; Yu et
15 al. Nature 396:469-474 1998).

16 The non-receptor protein tyrosine kinase Src is a
17 ubiquitous enzyme with key roles in diverse development,
18 physiological and pathological processes (Brown et al.
19 Biochim. Biophys. Acta 1287:121-149 1996). Domains identified
20 in Src-the Src homology 3 (SH3) domain, the SH2 domain and
21 the SH1 (catalytic) domain are signature regions that have
22 been used to define highly-conserved protein modules found in
23 a wide variety of signaling proteins (Pawson Nature 373:573-
24 580 1995). In addition to these highly-conserved regions, Src

1 also contains a region of low sequence conservation and
2 unknown function, termed the unique domain.

3 Src is highly expressed in the CNS, functioning to
4 regulate glutamatergic neurotransmission and synaptic
5 plasticity (Ali et al. Current Opinion in Neurobiology
6 11:336-342 2001). At glutamatergic synapses, Src modulates
7 the activity of NMDARs (Yu et al. Science 275:674-678 1997;
8 Lu et al. Science 279:1363-1368 1998; Yu et al. Nature
9 396:469-474 1998). Src represents a point through which
10 multiple signaling cascades from G-protein coupled receptors
11 (Luttrell et al. Journal of Cell Science 115:455-465 2002),
12 Eph receptors (Henderson et al. Neuron 32:1041-1056 2001;
13 Takasu et al. Science 295:491-495 2002; Murai et al. Neuron
14 33:159-162 2002) and integrins (Lin et al. Journal of
15 Neurophysiology 89:2874-2878 2003; Kramar et al. Journal of
16 Biological Chemistry 278:10722-10730 2003) converge to
17 upregulate NMDAR channel activity, thus mediating essential
18 neuronal excitation. The upregulation of NMDAR activity by
19 Src is necessary for long-term potentiation (LTP) of synaptic
20 transmission at Schaffer collateral-CA1 neuron synapses in
21 the hippocampus (Ali et al. Current Opinion in Neurobiology
22 11:336-342 2001), the predominant cellular model for learning
23 and memory (Kandel Science 294:1030-1038 2001).

24 However, abnormal regulation of NMDARs can have numerous

1 pathologic effects; most resulting from the production of
2 nitric oxide, a signaling molecule which mediates
3 excitotoxicity (Dawson et al. Proceedings of the National
4 Academy of Science USA 88:6368 1991). NMDARS mediate ischemic
5 brain injury, as seen, for example in stroke and traumatic
6 injury (Simon et al. Science 226:850 1984). In addition,
7 abnormal NMDAR regulation has been implicated in Alzheimer's
8 disease, Parkinson's disease (Coyle et al. Science 262:689
9 1993), schizophrenia (Hirsch et al. Pharmacology Biochemistry
10 and Behavior 56(4):797-802 1997), epilepsy (US Patent
11 5,914,403), glaucoma (US Application 2002 0077322 A1) and
12 chronic pain (Guo et al. Journal of Neuroscience 22:6208-6217
13 2002).

14 Although NMDARs are implicated in numerous pathological
15 conditions, non-selective blocking of their function is
16 deleterious, since complete blockade of synaptic transmission
17 mediated by NMDA receptors is known to hinder neuronal
18 survival (Ikonomidou et al. Lancet: Neurology 1:383-386 2002;
19 Fix et al. Experimental Neurology 123:204 1993; Davis et al.
20 Stroke 31:347 2000; Morris et al. Journal of Neurosurgery
21 91:737 1999).

22 Additionally, inhibition of Src kinases may also have
23 deleterious results. Since kinases play a part in the
24 regulation of cellular proliferation, they are frequently

1 targeted for the development of new cancer therapies.
2 The majority of these therapies inhibit function of the
3 kinase catalytic domain, which is often highly conserved
4 between distinct kinases. Thus, inhibition of Src in the CNS
5 with a standard kinase inhibitor may cross-react with
6 additional kinases and adversely affect normal neuronal
7 functions.

8 Considering the above-mentioned deleterious effects
9 resulting from direct blockage of NMDARs and/or indirect
10 inhibition of NMDARs through the use of kinase inhibitors, it
11 is clear that there remains a need in the art for a method of
12 modifying NMDARs which can attenuate downstream NMDAR
13 signaling, without completely blocking, ion-channel activity.
14

15 DESCRIPTION OF THE PRIOR ART

16 Since the NMDA receptor is critical to both normal
17 neuronal function and pathology, there are many known methods
18 for modification of NMDA receptors; several examples of which
19 are noted below.

20 US Patent 5,888,996 (David Farb) discloses a method for
21 inhibiting NMDA glutamate receptor-mediated ion channel
22 activity by treatment with an effective amount of a
23 derivative of pregnenolone sulfate. This patent also
24 discloses a method for modulating/altering excitatory

1 glutamate-mediated synaptic activity by contacting neurons
2 with pregnenolone sulfate or a derivative of pregnenolone
3 sulfate.

4 US Patent 5,914,403 (Nichols et al.) discloses agents
5 capable of modifying neuroexcitation through excitatory amino
6 acid antagonists; in particular quinolinic acid derivatives
7 antagonistic to a glycine binding site in the NMDAR complex.
8 The agents disclosed by Nichols et al. have anticonvulsant
9 activity.

10 US Patent 4,994,446 (Sokolovsky et al.) discloses a drug
11 system comprising a MK-801/PCP type drug administered in
12 combination with/or in sequence with excitatory amino acids
13 such as, glutamate, glycine, aspartate and analogs thereof.
14 The excitatory amino acids facilitate binding of the drug to
15 the NMDAR channels. This drug system has anticonvulsant
16 activity and can alleviate brain damage due to stroke.

17 US Patent 6,653,354 (Franks et al.) discloses a method
18 for reducing the level of NMDAR activation by use of the NMDA
19 antagonist, xenon to inhibit synaptic plasticity. The xenon
20 composition of Franks et al. also acts as a neuroprotectant.

21 US Patent Application 2002 0123510 A1 (Chenard et al.)
22 discloses a method for treatment of traumatic brain injury
23 (TBI) and stroke by administration of a NR2B subtype
24 selective NMDAR antagonist in combination with either of the

1 following agents; sodium channel antagonist, nitric oxide
2 synthase inhibitor, glycine site antagonist, potassium
3 channel opener, AMPA/kainate receptor antagonist, calcium
4 channel antagonist, GABA-A receptor modulator, anti-
5 inflammatory agent or a thrombolytic agent. These agents
6 either protect neurons from toxic insult, inhibit
7 inflammatory responses after brain damage or promote cerebral
8 reperfusion after hypoxia or ischemia.

9 Planells-Cases et al. (Mini Review of Medicinal
10 Chemistry 3(7):749-756 2003) disclose that small molecule
11 antagonists of the NMDAR are useful for the treatment of
12 neuropathic pain caused by injury to the peripheral or
13 central nervous system.

14 US Patent Application 2002 0077322 A1 (George Ayoub)
15 discloses methods for protecting neuronal cells from
16 glutamate-induced toxicity, such as that which occurs in
17 ischemia and glaucoma, by increasing the activity of a
18 cannabinoid agonist which binds specifically to a cannabinoid
19 receptor.

20 US Patent Application 2003 0050243 A1 (Michael
21 Tymianski) discloses a method for inhibition of binding
22 between NMDARs and neuronal proteins. The inhibition is
23 created by administration of a peptide replacement of either
24 an NMDAR or neuronal protein interaction domain. Post-

1 synaptic density protein 95 (PSD-95) couples NMDARs to
2 pathways mediating excitotoxicity and ischemic brain damage.
3 The method of Tymianski involves transducing neurons with
4 peptides that bind modular domains on either side of the
5 NMDAR/PSD-95 interaction complex. This transduction
6 attenuates downstream NMDAR signaling without blocking
7 receptor activity, protects cortical neurons from ischemic
8 insult and reduces cerebral infarction in rats exposed to
9 transient focal cerebral ischemia. This treatment was
10 effective in the rats when applied before or one hour after
11 the ischemic insult. (Aarts et al. Science 298:846-850 2002)
12 also discloses the research described in US Patent
13 Application 2003 0050243 A1.

14 As is exemplified by the examples listed above, the
15 majority of known methods for modification of NMDA receptors
16 generally involve administration of receptor antagonists
17 which inhibit receptor function completely. The instant
18 inventors are the first to modify the NMDAR by inhibiting the
19 interaction of the unique domain of the tyrosine kinase Src
20 enzyme with NADH dehydrogenase subunit 2 (ND2); thus
21 preventing Src upregulation of the NMDAR by preventing
22 binding between Src and ND2.

23

24

1 SUMMARY OF THE INVENTION

2 Src-mediated upregulation of NMDAR activity is prevented
3 by peptide fragments of the Src unique domain and by a unique
4 domain-binding antibody (Yu et al. Science 275:674-678 1997;
5 Lu et al. Science 279:1363-1368 1998) leading to the
6 hypothesis that the upregulation of NMDAR function by Src
7 depends on an interaction between a region in the unique
8 domain of Src and an unknown protein in the NMDAR complex
9 (Ali et al. Current Opinion in Neurobiology 11:336-342 2001).
10 In order to test the hypothesis, the instant inventors
11 searched for proteins that may interact with the unique
12 domain of Src and may thereby mediate the interaction between
13 this kinase and NMDARs. These proteins were generally termed
14 "SUDAPIs" (Src unique domain anchoring protein inhibitors) by
15 the instant inventors since they anticipate that other such
16 inhibitors may exist which exhibit identical functions.

17 As a result of their search, the instant inventors
18 became the first to identify NADH dehydrogenase subunit 2
19 (ND2; nucleotide sequence SEQ ID NO:8 and amino acid sequence
20 SEQ ID NO:9) as a Src unique domain-interacting protein. ND2
21 functions as an adapter protein anchoring Src to the NMDAR
22 complex, thus permitting Src-mediated upregulation of NMDAR
23 activity. The instant inventors identified a region of the
24 Src unique domain which interacts with ND2; a region located
25 approximately at amino acid positions 40-49 of the Src

1 protein(SEQ ID NO:1). The exogeneous peptide inhibits the
2 ability of ND2 to anchor the Src protein to the NMDAR
3 complex. This peptide, approximately 10 amino acids in
4 length, has been named "SUDAPI-1" by the instant inventors,
5 since it is the first such peptide discovered which functions
6 to inhibit the Src unique domain anchoring protein.
7 Administration of this exogeneous peptide prevents ND2
8 interaction with the Src unique domain; thus inhibiting Src-
9 mediated upregulation of NMDAR activity. Since this peptide
10 alone cannot cross the cell membrane to enter the cellular
11 interior, it is combined with a carrier capable of
12 penetrating the cell membrane. Illustrative, albeit non-
13 limiting examples of carriers are peptides derived from viral
14 transduction domains, such as the TAT domain derived from the
15 Human Immunodeficiency Virus (HIV) and VP22 derived from the
16 Herpes Simplex Virus, arginine-rich peptides, fusogenic
17 antennapedia peptides derived from Drosophila and lipids.
18 Lipids can facilitate crossing of the cell membrane by
19 enclosing the peptide in a lipid vesicle or liposome (lipid
20 transfection protocol) or the peptide can be directly
21 modified with lipid groups. Use of the HIV-Tat domain peptide
22 as a carrier is exemplified in the Examples described herein.
23 SUDAPI-1 fused to the HIV-Tat domain is designated "TSUDAPI-
24 1" (SEQ ID NO:2). The NMDAR activity is evoked by glutamate

1 and is additionally regulated by many distinct pathways other
2 than the Src pathway. Inhibition of Src suppresses but does
3 not completely inhibit the NMDAR as is apparent from the
4 electrophysiologic measurements of receptor activity shown in
5 Figures 5D-F. Thus, the instant invention provides methods
6 and compositions for modifying NMDAR function without
7 completely blocking the receptor or adversely affecting other
8 neuronal proteins with the use of generalized kinase
9 inhibitors. These methods and compositions may be used to
10 ameliorate diseases and/or other conditions related to NMDAR
11 signaling. Illustrative, albeit non-limiting examples of such
12 diseases and/or other conditions are stroke, hypoxia,
13 ischemia, multiple sclerosis, Huntington's chorea,
14 Parkinson's disease, Alzheimer's disease, hyperglycemia,
15 diabetes, traumatic injury, epilepsy, grand mal seizures,
16 spasticity, cerebral palsy, asthma, cardiac arrest, macular
17 degeneration, mental diseases, schizophrenia, AIDS dementia
18 complex, other dementias, AIDS wasting syndrome,
19 inflammation, pain, opioid addiction, cocaine addiction,
20 alcohol addiction, other conditions associated with substance
21 abuse and anorexia. An example of such amelioration is
22 illustrated in Example 7 wherein pain behaviors are reduced
23 in rats treated with the composition of the instant invention
24 prior to undergoing the formalin test.

1 Src upregulation of the NMDAR is involved in the pathway
2 of long-term potentiation (LTP) (Huang et al. Neuron 29:485-
3 496 2001; Lu et al. Science 279:1363-1367 1998). Since LTP is
4 considered a model for learning and memory, the compositions
5 of the instant invention are contemplated for use in methods
6 which elucidate mechanisms of learning and memory and/or
7 enhance learning and memory.

8 The NMDAR is expressed almost exclusively in neurons;
9 however the interaction between Src and ND2 was shown to
10 occur in multiple and diverse tissues (Example 8 and Figures
11 10A-B). Thus, the instant inventors hypothesize that the Src-
12 ND2 interaction has functions other than regulation of
13 NMDAR's and contemplate that the compositions of the instant
14 invention can be used in methods for the general inhibition
15 of Src in multiple cell types.

16 Accordingly, it is an objective of the instant invention
17 to provide a method for modifying NMDAR interaction with non-
18 receptor tyrosine kinase Src in any cell which expresses the
19 NMDAR by providing a composition including at least one
20 SUDAPI and administering the composition to the cell in an
21 amount effective to achieve modification of the NMDAR
22 interaction with non-receptor tyrosine kinase Src in the cell
23 wherein said modification ameliorates a disease or condition
24 related to NMDAR signaling. The methods and compositions of

1 the instant invention are particularly suited to use with
2 cells of the nervous system but can also be used with any
3 cell which expresses the NMDAR.

4 It is another objective of the instant invention to
5 provide a pharmaceutical composition for modifying NMDAR
6 interaction with non-receptor tyrosine kinase Src in cells
7 comprising at least one SUDAPI combined with a
8 pharmacologically acceptable solution or carrier.

9 It is also an objective of the instant invention to
10 provide a method for modifying NMDAR interaction with non-
11 receptor tyrosine kinase Src in any cell which expresses the
12 NMDAR by providing a composition including SUDAPI-1 and
13 administering the composition to the cell in an amount
14 effective to achieve modification of the NMDAR interaction
15 with non-receptor tyrosine kinase Src in the cell wherein
16 said modification ameliorates a disease or condition related
17 to NMDAR signaling.

18 It is another objective of the instant invention to
19 provide a pharmaceutical composition for modifying NMDAR
20 interaction with non-receptor tyrosine kinase Src in cells
21 comprising SUDAPI-1 combined with a pharmacologically
22 acceptable solution or carrier.

23 It is yet another objective of the instant invention to
24 provide a method for modifying NMDAR interaction with non-

1 receptor tyrosine kinase Src in any cell which expresses the
2 NMDAR by providing a composition including TSUDAPI-1 and
3 administering the composition to the cell in an amount
4 effective to achieve modification of the NMDAR interaction
5 with non-receptor tyrosine kinase Src in the cell wherein
6 said modification ameliorates a disease or condition related
7 to NMDAR signaling.

8 It is still another objective of the instant invention
9 to provide a pharmaceutical composition for modifying NMDAR
10 interaction with non-receptor tyrosine kinase Src in cells
11 comprising TSUDAPI-1 combined with a pharmacologically
12 acceptable solution.

13 It is another objective of the instant invention to
14 provide an isolated peptide (ND2.1; SEQ ID NO:7) which
15 interacts with the Src unique domain to anchor Src to the
16 NMDAR complex thus permitting Src-mediated upregulation of
17 NMDAR activity.

18 It is still another objective of the instant invention
19 to provide a method for inhibiting non-receptor tyrosine
20 kinase Src in cells expressing non-receptor tyrosine kinase
21 Src by providing a composition including at least one SUDAPI
22 and administering the composition to the cells in an amount
23 effective to achieve inhibition of non-receptor tyrosine
24 kinase Src in the cells.

1 It is another objective of the instant invention to
2 provide a pharmaceutical composition for inhibiting non-
3 receptor tyrosine kinase Src in cells comprising at least one
4 SUDAPI combined with a pharmacologically acceptable solution
5 or carrier.

6 It is another objective of the instant invention to
7 provide a composition useful in methods for elucidating the
8 mechanisms of learning and memory.

9 It is yet another objective of the instant invention to
10 provide a composition useful in methods for enhancing
11 learning and memory.

12 Other objectives and advantages of the instant invention
13 will become apparent from the following description taken in
14 conjunction with the accompanying drawings wherein are set
15 forth, by way of illustration and example, certain
16 embodiments of the instant invention. The drawings
17 constitute a part of this specification and include exemplary
18 embodiments of the present invention and illustrate various
19 objects and features thereof.

20

21 ABBREVIATIONS AND DEFINITIONS

22 The following list defines terms, phrases and
23 abbreviations used throughout the instant specification.

24 Although the terms, phrases and abbreviations are listed in

1 the singular tense the definitions are intended to encompass
2 all grammatical forms.

3 As used herein, the term "modification" refers to any
4 action and/or treatment which alters the function of a
5 protein.

6 As used herein, the term "inhibition" refers to any
7 action and/or treatment which operates against the full
8 activity of a protein thus reducing and/or completely
9 suppressing protein function.

10 As used herein, the term "interaction" refers to an
11 action wherein two substances in close physical proximity act
12 upon each other.

13 As used herein, the term "anchor" means to stabilize or
14 secure firmly in place.

15 As used herein, the term "isolated peptide" refers to a
16 peptide which has been "altered by the hand of man" and
17 separated from the co-existing materials of its natural
18 state. An isolated peptide has been changed or removed from
19 its original environment or both.

20 As used herein, the abbreviation "CNS" refers to the
21 central nervous system, which includes the brain, cranial
22 nerves and the spinal cord.

23 As used herein, the abbreviation "PNS" refers to the
24 peripheral nervous system, which is the network of nerves

1 that connect the CNS to organs, muscles, vessels and glands.

2 As used herein, the term "excitatory neurotransmission"
3 refers the passage of signals from one neuron to another via
4 chemical substances or electrical impulses.

5 As used herein, the abbreviation "NMDAR" refers to the
6 N-methyl-D-aspartate receptor, an ionotropic cation-ion
7 specific, ligand-gated(glutamate-gated) ion channel which is
8 activated by NMDA or NMDA-like ligands(agonist
9 activation)such as glutamate. The NMDAR is a multi-protein
10 complex including the core channel subunits with associated
11 scaffolding and regulatory proteins, located in the
12 excitatory synapses in the post-synaptic density. Activation
13 of the receptor opens the channel to allow cations (Ca^{+2} , Na^{+}
14 and K^{+})to cross the cellular membrane. "Upregulation of NMDAR
15 activity" refers to the enhanced opening of the receptor ion
16 channels.

17 As used herein, the abbreviation "PSD" refers to the
18 post-synaptic density, a specialized portion of the neuronal
19 cytoskeleton, located near the post-synaptic membrane. The
20 PSD provides a support matrix for signal transduction.

21 As used herein, the term "Src" refers to a protein
22 exhibiting tyrosine-specific kinase activity. The Src protein
23 is involved in controlling diverse cellular functions,
24 including regulation of NMDAR activity.

1 As used herein, the abbreviation "LTP" refers to long
2 term potentiation, an activity-dependent persistent
3 enhancement of synaptic transmission which is considered a
4 model of learning and memory. The biochemical signaling
5 cascade which results in LTP involves the activation of Src
6 which in turn, activates NMDARs.

7 As used herein, the abbreviation "ND2" refers to NADH
8 dehydrogenase subunit 2, a subunit of mitochondrial Complex
9 I. The instant inventor was the first to recognize that ND2
10 is present in the PSD and acts as an adaptor protein for
11 anchoring Src to the NMDAR complex.

12 As used herein, the abbreviation "SUDAPI" refers to any
13 substance which functions as a Src unique domain anchoring
14 protein inhibitor.

15 As used herein, the abbreviation "SUDAPI-1" refers to
16 the first Src unique domain anchoring protein inhibitor
17 discovered by the instant inventors. SUDAPI-1 is a peptide,
18 generally 10 amino acid residues in length corresponding
19 approximately to amino acid positions 40-49 of the Src unique
20 domain (SEQ ID NO:1).

21 As used herein, the phrase "corresponding approximately
22 to amino acid positions 40-49 of the Src unique domain"
23 refers to the slight difference which is possible in amino
24 acid position numbering of the Src protein due to species

1 variations and conventions within the art regarding whether
2 the first methionine counts as a residue or not.

3 As used herein, the abbreviation "TSUDAPI-1" refers to
4 SUDAPI-1 which is combined with the carrier peptide, HIV-Tat
5 (SEQ ID NO:2).

6 As used herein, the term "carrier" refers to any
7 substance which is attached to another substance which alone
8 cannot traverse the cell membrane to enter the cellular
9 interior. The carrier substance functions to carry this other
10 substance through the cellular membrane into the cellular
11 interior. Illustrative, albeit non-limiting examples include
12 lipids and peptides having transducing and/or fusogenic
13 ability.

14 As used herein, the term "HIV-Tat" refers to the
15 transduction domain of the human immunodeficiency virus
16 (HIV); the causative agent of Acquired Immunodeficiency
17 Syndrome (AIDS). HIV-Tat peptide is often used as a carrier
18 to transport molecules into cells.

19 As used herein, the term "VP22" refers to a transduction
20 domain of the herpes simplex virus. VP22 peptide is often
21 used as a carrier to transport molecules into cells.

22 As used herein, the term "antennapedia" refers to
23 peptides derived from Drosophila which have fusogenic
24 ability. Antennapedia peptide is often used as a carrier to

1 transport molecules into cells.

2 The phrase "pharmaceutically acceptable" is used herein
3 as described in US 6,703,489. "Pharmaceutically acceptable"
4 means approved by a regulatory agency or listed in a
5 generally approved pharmacopeia for use in animals and
6 humans. Solutions are usually preferred when a composition is
7 administered intravenously. Illustrative, albeit non-limiting
8 examples of pharmaceutically acceptable solutions include
9 water, oils, saline, aqueous dextrose and glycerol.

10 As used herein, the phrase "amount effective" refers to
11 an amount of a composition sufficient to elicit a change in
12 activity of the NMDAR.

13 As used herein, the phrase "ameliorate a disease and/or
14 condition" refers to an action which causes symptoms of a
15 disease and/or condition to improve or become better.

16 As used herein, the abbreviation "SH" refers to a Src
17 homology domain; regions that have been used to define
18 highly-conserved protein modules found in a wide variety of
19 signaling proteins (T. Pawson Nature 373:573-580 1995).

20 As used herein, the phrase "unique domain" refers to a
21 Src domain having low sequence conservation and unknown
22 function.

23 As used herein, the abbreviation "ND4" refers to NADH
24 dehydrogenase subunit 4, an oxidoreductase protein, a

1 component of mitochondrial Complex I (JE Walker Quarterly
2 Reviews of Biophysics 25(3):253-324 1992; Sazanov et al.
3 Biochemistry 39:7229-7235 2000; Sazanov et al. Journal of
4 Molecular Biology 302:455-464 2000).

5 As used herein, the abbreviation "Cyto 1" refers to
6 cytochrome *c* oxidase subunit 1, an inner mitochondrial
7 membrane protein that is part of Complex IV (Marusich et al.
8 Biochim. Biophys. Acta 1362:145-159 1997).

9 As used herein, the abbreviation "mEPSCs" refers to
10 miniature excitatory post-synaptic currents, a type of
11 excitatory neurotransmission.

12 The terms "SUDAPI-1" and "Src40-49" are used
13 interchangeably herein (SEQ ID NO:1).

14 The terms "TSUDAPI-1"; "Src40-49-Tat"; "Src40-49-HIV-
15 Tat"; "Tat-Src40-49" and "HIV-Tat-Src40-49" are used
16 interchangeably herein (SEQ ID NO:2).

17 The terms "Src40-58" and "scrambled Src40-58" are used
18 repeatedly throughout and refer to peptides comprising amino
19 acid residues 40-58 of SEQ ID NO:4.

20 The term "Src49-58" is used repeatedly throughout and
21 refers to a peptide comprising amino acid residues 49-58 of
22 SEQ ID NO:4.

1 BRIEF DESCRIPTION OF THE FIGURES

2 Figures 1A-E show the results of experiments evidencing
3 that ND2 is a Src unique domain-interacting protein.

4 Figures 2A-E show the results of experiments evidencing
5 that ND2 is present at the post-synaptic density.

6 Figures 3A-B show the results of experiments evidencing
7 that ND2 interacts with Src at the post-synaptic density.

8 Figures 4A-G show the results of experiments evidencing
9 that ND2 interacts with Src at the NMDAR complex.

10 Figures 5A-F show the results of experiments evidencing
11 that blocking expression of ND2 prevents Src-dependent
12 regulation of NMDA receptor activity. Figure 5F shows amino
13 acid residues 4-7 of SEQ ID NO:5 (pY)EEI.

14 Figures 6A-C show the results of experiments evidencing
15 that the Src40-49 (SUDAPI-1) peptide specifically interacts
16 with the ND2.1 peptide.

17 Figures 7A-D show results of experiments showing the
18 effects of TSUDAPI-1 on 2.5% formalin induced flinching or
19 biting/licking behaviors.

20 Figures 8A-D show results of experiments showing the
21 effects of HIV-TAT on 2.5% formalin induced flinching or
22 biting/licking behaviors.

23 Figures 9A-B show SEQ ID NOS:6 and 7; Figure 9A shows
24 the nucleotide sequence encoding recombinant ND2.1

1 protein(SEQ ID NO:6); Figure 9B shows the amino acid sequence
2 of recombinant ND2.1 protein (SEQ ID NO:7).

3 Figures 10A-B show immunoblots evidencing that ND2 and
4 Src interact in multiple, diverse tissues.

5

6 DETAILED DESCRIPTION OF THE INVENTION

7

8 EXAMPLE 1

9 NADH dehydrogenase subunit 2 (ND2) is a Src unique
10 domain-binding protein.

11

12 A yeast two-hybrid screen of a fetal brain library using
13 bait constructs containing the murine Src unique domain was
14 conducted in order to search for proteins that interact with
15 the Src unique domain.

16 cDNAs encoding amino acids 4-82 (the Src unique domain)
17 and amino acids 4-150 (the Src unique and SH3 domains) of
18 murine n-Src were ligated into pEG202 (Gyuris et al. Cell
19 75:791-803 1993) to create two expression vectors encoding in
20 frame LexA fusions containing the Src unique domain (the
21 nucleotide sequence encoding Src is SEQ ID NO:3 and the amino
22 acid sequence is SEQ ID NO:4). The bait constructs were then
23 sequenced. Both baits were tested to ensure that the baits
24 did not activate transcription of the reporters in the

1 absence of prey and that both could enter the nucleus and
2 bind to LexA operators. To create the selection strains for
3 screening, each bait plasmid was individually transformed
4 into the yeast strain EGY48. EGY48 has an integrated Leu2
5 selectable marker regulated by 6 LexA operator repeats, and
6 carries a reporter plasmid with the lacZ gene regulated by 8
7 LexA operator repeats. Bait-prey interactions that occur
8 with low affinity result in activation of the Leu2 reporter
9 gene only, whereas high affinity interactions result in
10 activation of both the Leu2 and lacZ reporter genes, allowing
11 for double selection of prey. The selection strain was
12 transformed with a representative activation-tagged cDNA prey
13 fusion library constructed using ~1 kilobase EcoRI fragmented
14 poly A(+) RNA from human fetal brain. Yeast transformed with
15 the prey library (approximately 1.1×10^6 clones) were
16 screened by double selection on X-gal Leu⁻ medium. Prey
17 cDNAs encoding proteins that interacted with the bait were
18 isolated and sequenced.

19 Src, Fyn, and ND2 recombinant proteins were prepared.
20 The cDNAs encoding the SH3 and SH2 domains of mouse n-Src and
21 Fyn were PCR subcloned, ligated in frame into pGEX4T-1
22 (Amersham Pharmacia Biotech, Baie d'Urfé, Québec), and
23 sequenced. These plasmids, as well as plasmids encoding the
24 unique domains of Src and Fyn in pGEX2T'6, were transformed

1 into BL21 bacteria, and GST fusion proteins were purified by
2 glutathione affinity chromatography. To create the ND2.1,
3 ND2.2, and ND2.3 GST fusion proteins, cDNAs encoding amino
4 acids 239-321 (ND2.1-GST; SEQ ID NO:7), amino acids 189-238
5 (ND2.2-GST; SEQ ID NO:11), and amino acids 1-188 (ND2.3-GST;
6 SEQ ID NO:13) of human ND2 were PCR subcloned and ligated
7 into pGEX4T-1 (the nucleotide sequence encoding ND2 is SEQ ID
8 NO:8 and the amino acid sequence is SEQ ID NO:9; the
9 nucleotide sequences encoding ND2.1; ND2.2 and ND2.3 are SEQ
10 ID NOS:6, 10 and 12, respectively). Using PCR-based single
11 nucleotide mutagenesis, all cDNAs encoding ND2 fusion
12 proteins were corrected for differences between mitochondrial
13 and nuclear codons to prevent premature translation
14 termination and protein truncation. All constructs were then
15 confirmed by sequencing. The plasmids were transformed into
16 bacteria, and GST fusion proteins were purified by
17 glutathione affinity chromatography.

18 Detailed protocols for in vitro binding assays, pull
19 down assays, immunoblotting, and co-immunoprecipitation
20 techniques can be found in Pelkey et al. (Neuron 34:127-138
21 2002).

22 In two independent screens, cDNA fragments encoding
23 overlapping regions within NADH dehydrogenase subunit 2 (ND2)
24 were isolated (Figure 1A). ND2 is a 347 amino acid protein

(SEQ ID NO:9) that is a subunit of the inner mitochondrial membrane enzyme, NADH dehydrogenase (Complex I). ND2 is one of a group of seven oxidoreductase proteins that are encoded in the mitochondrial genome and which co-assemble with 35 nuclear encoded subunits to form Complex I. ND2 on its own lacks enzymatic activity (J.E. Walker Quarterly Reviews of Biophysics 25(3):253-324 1992; Sazanov et al. Journal of Molecular Biology 302:455-464 2000; Sazanov et al. Biochemistry 39:7229-7235 2000). Figure 1A is a schematic diagram illustrating the domain structure of ND2, clones isolated from the yeast two hybrid screen, and recombinant GST-tagged fusion proteins. The lines point out the beginning of the oxidoreductase domain at amino acid position 23 and the end at amino acid position 197. Each clone and GST-fusion protein represent overlapping regions within ND2.

As yeast two-hybrid screening may reveal false positive protein-protein interactions, the interaction between Src and ND2 was observed using an independent methodological approach. Direct binding in vitro between ND2 and Src was tested using recombinant proteins. A series of GST fusion proteins comprised of portions of ND2 that spanned the overlapping region found with the yeast two-hybrid screen were made (Figure 1A). Importantly, the cDNAs encoding each of the ND2 fusion proteins were corrected for differences

1 between mitochondrial and nuclear codons so that the sequence
2 of the ND2 portion of the fusion proteins was that which
3 would have been produced by translation in the mitochondria.
4 For example, Figure 9A shows the nucleotide sequence encoding
5 recombinant ND2.1 protein (SEQ ID NO:6). Codons that are
6 highlighted with bold type were altered by PCR-based single
7 nucleotide mutagenesis. TGA was changed to TGG to prevent
8 premature translation termination and protein truncation. GAA
9 was changed to GAG to remove a restriction enzyme site.
10 Numbers in parenthesis correspond to equivalent positions in
11 the endogenous human ND2 nucleotide sequence. Figure 9B
12 shows the amino acid sequence of recombinant ND2.1
13 protein(SEQ ID NO:7). Numbers in parenthesis correspond to
14 equivalent positions in the endogenous human ND2 amino acid
15 sequence. Each of the series of GST-fusion proteins was
16 tested individually for interaction with the Src unique
17 domain ("pull-down" assay). Figure 1B shows a blot of ND2-
18 GST fusion proteins probed with biotinylated Src unique
19 domain followed by a streptavidin-HRP conjugate. A GST
20 fusion protein containing amino acids 239-321 of ND2 (ND2.1-
21 GST; SEQ ID NO:7) was found that bound to the unique domain
22 of Src (Figure 1B). In contrast, GST fusion proteins
23 containing amino acids 189-238 (ND2.2-GST) or 1-188 (ND2.3-
24 GST) of ND2 (ND2 protein sequence is SEQ ID NO:9) did not

1 bind to the Src unique domain. These results, together with
2 those from the yeast two-hybrid screen, indicate that ND2 is
3 a Src unique domain-binding protein. The results indicate
4 further that the Src-binding portion of ND2 is contained
5 within the region of amino acids 239-321 (SEQ ID NO:7). This
6 region of ND2 shows little conservation amongst the
7 mitochondrially encoded oxidoreductase proteins and is
8 outside the so-called "oxidoreductase domain", a signature
9 region identified in all mitochondrially encoded subunits of
10 NADH dehydrogenase (J.E. Walker Quarterly Reviews of
11 Biophysics 25(3):253-324 1992; Sazanov et al. Journal of
12 Molecular Biology 302:455-464 2000; Sazanov et al.
13 Biochemistry 39:7229-7235 2000) and some antiporters
14 (Fearnley et al. Biochim. Biophys. Acta 1140:105-143 1992).

15 Another "pull-down" assay was conducted to determine
16 whether the binding of ND2 might generalize to other domains
17 of Src or to other Src family tyrosine kinases.

18 However, it was found that ND2.1-GST did not bind to
19 either of the prototypic protein-protein interaction domains
20 of Src, the SH2 or SH3 domains (Figure 1C). Figure 1C shows a
21 blot of ND2.1-GST probed with biotinylated domains of Src and
22 Fyn followed by streptavidin-HRP conjugate.

23 To examine the potential interaction of ND2 with other
24 kinases of the Src family recombinant domains of Fyn were

1 tested, the protein most closely related to Src but which has
2 little primary sequence conservation in the unique domain
3 (Brown et al. Biochim. Biophys. Acta 1287:121-149 1996; T.
4 Pawson Nature 373:573-580 1995). It was found that ND2.1-GST
5 did not interact in vitro with the Fyn unique domain; nor did
6 ND2.1 bind to the SH2 or SH3 domains of Fyn. Thus, the ND2.1
7 region does not interact with the SH2 or SH3 domains of Src
8 or Fyn nor does it generally bind to the unique domain of Src
9 family tyrosine kinases.

10 To investigate the possibility that Src and ND2 may
11 interact in vivo, brain lysates were immunoprecipitated with
12 antibodies directed against ND2 (anti-ND2) or against Src
13 (anti-Src). It was found that immunoprecipitating Src led to
14 co-immunoprecipitation of ND2 (Figure 1D). Figure 1D shows
15 immunoblots of co-immunoprecipitates from brain homogenate
16 probed with anti-ND2, anti-Src or anti-Fyn as indicated. Non-
17 specific IgG was used as a negative control for
18 immunoprecipitation. Fyn was readily detected in the brain
19 homogenate used as a starting material for the co-
20 immunoprecipitation (data not illustrated). Conversely,
21 immunoprecipitating with anti-ND2 resulted in co-
22 immunoprecipitation of Src. In contrast, anti-ND2 did not
23 co-immunoprecipitate Fyn and neither ND2 nor Src was
24 immunoprecipitated with a non-specific IgG (Figure 1D). As

1 an independent immunoprecipitation control it was found that
2 ND2 was co-immunoprecipitated by anti-Src from Src^{+/+}
3 fibroblasts but not from Src^{-/-} fibroblasts (Figure 1E).
4 Figure 1E shows an immunoblot of co-immunoprecipitates from
5 cultured Src^{+/+} and Src^{-/-} fibroblasts probed with anti-ND2.
6 Non-specific IgG was used as a negative control for
7 immunoprecipitation, and immunoblotting of ND2 protein from
8 both cell lines was used as a positive control. Thus, in
9 addition to finding the ND2-Src unique domain interaction in
10 two yeast two-hybrid screens and in vitro binding assays with
11 recombinant proteins, it was found that ND2 and Src co-
12 immunoprecipitated with each other, which together led to the
13 conclusion that the ND2 is a Src unique-domain binding
14 protein that may interact with Src in vivo.

15

16 EXAMPLE 2

17

18 ND2 is present in post-synaptic densities in brain.

19

20 Post-synaptic density proteins (Kennedy et al.
21 Proceedings of the National Academy of Science USA 80:7357-
22 7361 1983) were prepared from rat brain as described in
23 detail (Pelkey et al. Neuron 34:127-138 2002). Cellular
24 fractionation of rat brain tissue into nuclear, heavy

1 mitochondrial, light mitochondrial, microsomal, and cytosolic
2 fractions was performed by differential centrifugation of
3 tissue homogenate in 0.25 M sucrose/10 mM HEPES-NaOH, 1 mM
4 EDTA, pH 7.4 with 2 µg each of aprotinin, pepstatin A, and
5 leupeptin (Sigma, St. Louis, MO) at 4°C. Nuclei were
6 pelleted by centrifugation at 1 000 g for 10 minutes, the
7 supernatant was removed and spun at 3 000 g for 10 minutes to
8 obtain a heavy mitochondrial pellet. The supernatant was
9 removed and spun at 16 000 g for 15 minutes to obtain a light
10 mitochondrial pellet. The supernatant was removed and spun
11 at 100 000 g for 1 hour to obtain a microsomal pellet and the
12 cytosolic fraction. All pellets were then resuspended in
13 RIPA buffer (50 mM Tris pH 7.6, 150 mM NaCl, 1 mM EDTA, 1%
14 NP-40, 2.5 mg/ml NaDOC, 1 mM Na₃VO₄, 1 mM PMSF, and 2 µg/ml
15 each of protease inhibitors). The light mitochondrial
16 fraction was used in subsequent experiments. For Western
17 blots, 50 µg of total protein was loaded per lane, resolved
18 by SDS-PAGE, transferred to nitrocellulose membranes, and
19 probed with anti-ND2, anti-Cyto1 and anti-ND4 (mouse
20 monoclonals, Molecular Probes Inc., Eugene, OR), anti-PSD95
21 (mouse monoclonal clone 7E3-1B8, Oncogene Research Products,
22 Cambridge, MA), anti-NR1 (mouse monoclonal clone 54.1,
23 Pharmingen), anti-Src, or anti-synaptophysin (mouse
24 monoclonal, Sigma).

1 Post-embedding immunogold electron microscopy was
2 carried out. Sprague Dawley rats were anesthetized and
3 perfused with 4% paraformaldehyde plus 0.5% glutaraldehyde in
4 0.1 M phosphate buffer. Parasagittal sections of the
5 hippocampus were cryoprotected in 30% glycerol and frozen in
6 liquid propane. Frozen sections were immersed in 1.5% uranyl
7 acetate in methanol at -90°C, infiltrated with Lowicryl HM-20
8 resin at -45°C, and polymerized with ultraviolet light.
9 Sections were incubated in 0.1% sodium borohydride plus 50 mM
10 glycine in TBS and 0.1% Triton X-100 (TBST), followed by 10%
11 normal goat serum (NGS) in TBST, primary antibody in 1% NGS
12 in TBST, and immunogold (10 nm; Amersham Pharmacia Biotech)
13 in 1% NGS in TBST plus 0.5% polyethylene glycol. Finally,
14 the sections were stained in uranyl acetate and lead citrate
15 prior to analysis.

16 In the CNS a prominent subcellular location for Src is
17 in the post-synaptic density (PSD) (Yu et al. Science
18 275:674-678 1997), a subsynaptic specialization at
19 glutamatergic synapses comprised of
20 α -amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA-) and
21 NMDA-type glutamate receptors together with scaffolding,
22 signaling and regulatory proteins (Walikonis et al. Journal
23 of Neuroscience 20:4069-4080 2000). Because Src is known to
24 regulate subsynaptic NMDARs (Yu et al. Science 275:674-678

1 1997), if ND2 is the protein mediating the interaction
2 between NMDARs and the unique domain of Src then ND2 is
3 predicted to be present in the PSD. This was tested by
4 preparing PSD proteins from rat brain homogenates by
5 sequential fractionation and determining whether ND2 was
6 present in this fraction. Characteristic of a bona fide PSD
7 fraction, the fraction which was prepared contained post-
8 synaptic proteins including PSD-95 and NMDA receptor subunit
9 proteins but lacked the pre-synaptic protein synaptophysin
10 (Figure 2A). Figure 2A shows immunoblots of PSD proteins
11 probed with anti-ND2, anti-cytochrome c oxidase I (Cyto 1),
12 anti-ND4, anti-PSD95, anti-NR1, anti-Src and anti-
13 synaptophysin as indicated. It was found that ND2 was present
14 in the PSD fraction and the amount of ND2 estimated in this
15 fraction was approximately 15% of that in the total brain
16 homogenate. In contrast to ND2, neither the oxidoreductase
17 protein ND4, another mitochondrially-encoded component of
18 Complex I (J.E. Walker Quarterly Reviews of Biophysics
19 25(3):253-324 1992; Sazanov et al. Journal of Molecular
20 Biology 302:455-464 2000; Sazanov et al. Biochemistry
21 39:7229-7235 2000) nor cytochrome c oxidase subunit 1 (Cyto
22 1), an inner mitochondrial membrane protein that is part of
23 Complex IV (Marusich et al. Biochim. Biophys. Acta 1362:145-
24 159 1997), was detectable in the PSD fraction. On the other

1 hand, Cyto 1 and ND4, as well as ND2, were readily detected
2 in proteins from brain mitochondria (Figure 2B). Figure 2B
3 shows immunoblots of mitochondrial proteins prepared by
4 differential centrifugation probed with anti-ND2, anti-Cyto 1
5 and anti-ND4. Neither NR1 nor NR2A/B was detected in the
6 mitochondrial fraction (data not shown). Although the
7 molecular size of the protein detected by anti-ND2 in the PSD
8 preparation matched that of ND2 in mitochondria, it is
9 conceivable that the protein detected in the PSD preparation
10 was not ND2 but a protein of the same molecular size that was
11 recognized by anti-ND2. However, it was found that
12 incubating anti-ND2 with the antigen to which the antibody
13 was raised prevented the immunoblotting signal (Figure 2C).
14 Figure 2C shows immunoblots of PSD proteins showing the
15 specificity of the N-terminal ND2 antibody by pre-adsorption
16 with the antigenic peptide used to derive the antibody.
17 Moreover, it was found that a separate antibody directed
18 towards a distinct epitope in a region of ND2 remote from
19 that of the anti-ND2 epitope also detected ND2, at the
20 correct molecular size, in the PSD preparation, as well as in
21 the mitochondrial preparation (Figure 2D). Figure 2D shows
22 immunoblots of PSD and mitochondrial proteins probed with two
23 independent rabbit polyclonal antibodies directed against two
24 disparate regions of ND2. The N-terminal ND2 antibody was

1 used for all subsequent experiments illustrated. Thus, ND2
2 was found in the PSD preparation by two separate antibodies,
3 and this could not be accounted for by a general
4 contamination with mitochondrial proteins because neither
5 Cyto 1 nor ND4 were detected in the PSD.

6 In addition to examining PSD protein preparations, the
7 presence of ND2 in PSDs was tested for by means of post-
8 embedding immunogold electron microscopy in the CA1 stratum
9 radiatum of rat hippocampus (Petrálie et al. Nature
10 Neuroscience 2:31-36 1999; Sans et al. Journal of
11 Neuroscience 20:1260-1271 2000). With this experimental
12 approach the tissue is fixed immediately after the animal is
13 sacrificed and prior to sectioning so that protein
14 localization is preserved. ND2 labeling was found, as
15 visualized by secondary antibody conjugated to 10 nm gold
16 particles, in the PSD and the postsynaptic membrane in
17 dendritic spines of CA1 neurons (Figure 2E), as well as over
18 mitochondria (not illustrated).. Figure 2E shows three
19 representative post-embedding immunogold electron microscopy
20 images of rat hippocampus CA1 synapses, pre-synaptic. Scale
21 bar is 200nm. ND2 labeling was enriched in the post-synaptic
22 membrane approximately 30-fold as compared with the plasma
23 membrane in the remainder of the dendritic spine (0.37
24 particles per PSD/section versus 0.012, $p < 0.05$) and there was

1 no obvious accumulation of ND2 labeling along the plasma
2 membrane of the dendritic shaft. The ND2 labeling observed
3 in the PSD and post-synaptic membrane could not have been due
4 to labeling in mitochondria because it is known that
5 mitochondria are excluded from dendritic spines (Shepherd et
6 al. Journal of Neuroscience 18(20):8300-8310 1998). Thus,
7 these results indicate that ND2 is present in the
8 biochemically defined PSD protein fraction and is localized
9 at PSDs in CA1 neurons.

10

11 EXAMPLE 3

12

13 ND2 interacts with Src at the NMDA receptor complex in
14 post-synaptic densities.

15

16 Since previous results indicate that ND2 is present in
17 PSDs from brain, it was examined whether ND2 interacts with
18 Src in PSDs. It was found that immunoprecipitating ND2 from
19 the PSD fraction led to co-immunoprecipitation of Src and
20 vice versa (Figure 3A), indicating that ND2 and Src interact
21 post-synaptically at glutamatergic synapses. Figure 3A shows
22 immunoblots of co-immunoprecipitates from PSD preparations
23 probed with anti-ND2 or anti-Src as indicated. Non-specific
24 IgG (either rabbit or mouse) was used as a negative control

1 for both antibodies. Moreover, Src was pulled from the PSD
2 fraction by the fusion protein ND2.1-GST, but not by either
3 ND2.2- or ND2.3-GST (Figure 3B). Figure 3B shows recombinant
4 ND2.1-GST fusion protein, but not ND2.2-GST, ND2.3-GST, or
5 GST alone, pulls Src from PSD preparations. Thus, as it was
6 found with the Src-ND2 binding in vitro, these results
7 indicate that amino acids 239-321 of ND2 (SEQ ID NO:7) are
8 both necessary and sufficient for ND2 to interact with Src in
9 the PSD.

10 The hypothesis that ND2 is the protein mediating the
11 interaction between Src and NMDARs requires that, in addition
12 to being present in the PSD and interacting there with Src,
13 ND2 is part of NMDAR complex of proteins. To determine
14 whether ND2 is a component of the NMDAR protein complex,
15 NMDAR complexes were immunoprecipitated from the PSD
16 fraction, using an antibody directed against the core NMDAR
17 subunit NR1 (Dingledine et al. Pharmacology Reviews 51:7-61
18 1999), and the co-immunoprecipitating proteins were probed
19 with anti-ND2. It was found that ND2 co-immunoprecipitated
20 (Figure 4A), and conversely, immunoprecipitating with anti-
21 ND2 led to co-immunoprecipitation of NR1 (Figure 4A). Figure
22 4A shows immunoblots of co-immunoprecipitates from PSD
23 preparations probed with anti-ND2 or with anti-NMDA receptor
24 subunit 1 (NR1) as indicated. Non-specific IgG (either rabbit

1 or mouse) was used as a negative control for both antibodies.
2 Neither ND2 nor NR1 was immunoprecipitated by non-specific
3 IgG, and ND2 did not co-immunoprecipitate with the potassium
4 channel Kv3.1 (Figure 4B), a negative control for non-
5 specific immunoprecipitation of post-synaptic proteins,
6 therefore it was concluded that ND2 is an NMDAR complex
7 protein. Figure 4B shows an immunoblot of co-
8 immunoprecipitates from PSD preparations using anti-GluR2,
9 anti-GABA_AR α , anti-GABA_AR β 2/3 and anti-Kv3.1 antibodies to
10 immunoprecipitate. Probe was anti-ND2. Importantly, neither
11 ND4 nor Cyto 1 was detected in co-immunoprecipitates of NR1
12 (not illustrated) indicating that mitochondrial proteins in
13 general are not components of the NMDAR complex. Moreover,
14 ND2 did not co-immunoprecipitate with GluR2, GABA_AR α or GABA_A
15 R β 2/3 (Figure 4B) indicating that ND2 is not a detectable
16 component of AMPA receptor or γ -aminobutyric acid (GABA)
17 receptor complexes.
18 Thus, while ND2 is a component of NMDAR complexes it is not
19 generally a component of neurotransmitter receptor complexes
20 in the brain.

1 EXAMPLE 4

2

3 ND2 acts as an adapter protein for Src.

4

5 Src40-58 and scrambled Src peptides were biotinylated by
6 incubating with Sulfo-NHS-Biotin (Pierce Chemical Co.,
7 Rockford, IL) for 30 minutes at room temperature (SEQ ID
8 NO:4, Src protein). The biotinylation reaction was then
9 quenched by the addition of Tris-HCl (pH 8.0) to a final
10 concentration of 20 mM. Purified recombinant fusion proteins
11 (~20 µg each) were dotted onto nitrocellulose and dried
12 overnight. Membranes were blocked with 5% BSA in PBS for 1
13 hour, after which biotinylated peptides (30 µg/ml) diluted
14 1:1000 in fresh 5% BSA in PBS were added. The membranes were
15 incubated with the peptides for 1 hour, washed, and probed
16 using a streptavidin-HRP conjugate. Bound probe was then
17 detected on film using an ECL kit.

18

19 ND2 acts as an adapter protein for Src. Amino acids 40-
20 58 (SEQ ID NO:4) within the Src unique domain have been
21 implicated in the binding of Src to the interacting protein
22 in the NMDAR complex (Yu et al. Science 275:674-678 1997; Lu
23 et al. Science 279:1363-1368 1998; Yu et al. Nature 396:469-
24 474 1998) and thus, ND2 was predicted to bind to this region

1 of Src. This prediction was examined in vitro using a peptide
2 with the sequence of amino acids 40-58 (Src40-58;SEQ ID NO:4)
3 which was found to bind directly to ND2.1-GST (Figure 4C) in
4 vitro. In contrast, a peptide with identical amino acid
5 composition, but a scrambled sequence (scrambled Src40-58),
6 did not bind to ND2.1-GST. Neither Src40-58 nor scrambled
7 Src40-58 bound to ND2.2-GST, ND2.3-GST or to GST alone
8 (Figure 4C). Figure 4C shows a dot blot of ND2-GST fusion
9 proteins probed with biotinylated Src40-58 or scrambled
10 Src40-58 peptides followed by streptavidin-HRP conjugate.
11 Furthermore, the effect of Src40-58 on the interaction
12 between Src and ND2 was examined(Figures 4D and 4E). It was
13 found that incubating ND2.1-GST with Src40-58 prevented this
14 fusion protein from pulling down the Src unique domain
15 protein in vitro (Figure 4D). Figure 4D shows a blot of
16 ND2.1-GST probed with boptinylated Src unique domain in the
17 presence of either Src40-58 or scrambled Src40-58 peptides
18 followed by streptavidin-HRP conjugate. On the other hand,
19 scrambled Src40-58 did not affect the interaction between the
20 ND2.1-GST and Src unique domain proteins. Incubating PSD
21 proteins with Src40-58 prevented the co-immunoprecipitation
22 of ND2 by anti-Src but this was not affected by scrambled
23 Src40-58 (Figure 4E). Figure 4E shows immunoblots of co-
24 immunoprecipitates obtained from PSD proteins in the presence

1 of either Src40-58 or scrambled Src40-58 probed with anti-ND2
2 or stripped and re-probed with anti-Src. Importantly, Src40-
3 58 did not affect the immunoprecipitation of Src from PSDs.
4 Thus, it was concluded that amino acids 40-58 of Src interact
5 with the region spanned by ND2.1, thereby mediating the
6 binding between the Src unique domain and ND2.

7 As ND2 alone is not catalytically active (J.E. Walker
8 Quarterly Reviews of Biophysics 25(3):253-324 1992; Sazanov
9 et al. Journal of Molecular Biology 302:455-464 2000;
10 Sazanove et al. Biochemistry 39:7229-7235 2000), its
11 functional role in the NMDAR complex was investigated. ND2
12 might be a phosphorylation target for Src, but it was found
13 that ND2 immunoprecipitated from PSD protein fractions was
14 not detectably phosphorylated on tyrosine. Moreover,
15 inclusion of ND2.1-GST did not alter the catalytic activity
16 of Src in vitro (not illustrated) consistent with the binding
17 of ND2 to the unique domain rather than to the regulatory or
18 catalytic domains. Thus, it is unlikely that ND2 is a target
19 of Src or a regulator of Src kinase activity.

20 However, it was found that the co-immunoprecipitation of
21 Src with NMDARs (Figure 4F, left panel) was suppressed by
22 Src40-58, but not scrambled Src40-58, and by ND2.1 (Figure
23 4F, right panel) indicating that the association of Src with
24 the NMDAR complexes depends on the interaction with ND2.

1 Figure 4F, left panel shows immunoblots of co-
2 immunoprecipitates obtained from PSD proteins in the presence
3 of either Src40-58 or scrambled Src40-58. Figure 4F, right
4 panel shows immunoblots of co-immunoprecipitates obtained
5 from PSD proteins in the presence of GST-ND2.1 fusion protein
6 probed with anti-Src or anti-NR1 as indicated. In contrast,
7 the co-immunoprecipitation of ND2 with NMDARs was not
8 affected by Src40-58 (Figure 4G), implying that binding ND2
9 to Src is not necessary for ND2 to associate with NMDAR
10 complexes. Figure 4G shows immunoblots of co-
11 immunoprecipitates obtained from PSD proteins in the presence
12 of either Src40-58 or scrambled Src40-58 peptides probed
13 with anti-ND2 or stripped and re-probed with anti-NR1. Taking
14 these results together, it was concluded that ND2 may
15 function as an adapter protein that anchors Src in the NMDAR
16 complex.

17

18 EXAMPLE 5

19

20 Loss of ND2 in neurons prevents the regulation of NMDA
21 receptor activity by Src.

22

23 Fetal rat hippocampal neurons were prepared, cultured,
24 and used for electrophysiological recordings 12-17 days after

1 plating. Methods for whole cell recordings are described in
2 Pelkey et al. (Neuron 34:127-138 2002).

3
4
5 It was hypothesized that if ND2 is a Src adapter protein
6 then loss of ND2 should prevent the upregulation of NMDAR
7 activity by endogenous Src (Yu et al. Science 275:674-678
8 1997). This was tested by investigating miniature excitatory
9 post-synaptic currents (mEPSCs) recorded from cultured
10 hippocampal neurons (MacDonald et al. Journal of Physiology
11 (London) 414:17-34 1989). In these neurons the NMDAR-
12 mediated component of mEPSCs is increased by activating
13 endogenous Src with a high-affinity activating phosphopeptide
14 EPQ(pY)EEIPIA (Liu et al. Oncogene 8:1119-1126 1993) and is
15 reduced by applying Src40-58 (Yu et al. Science 275:674-678
16 1997). It is predicted that each of these effects will be
17 lost by blocking the expression of ND2, if it acts as an
18 adapter protein for Src in the NMDAR complex. In order to
19 suppress ND2 expression, the hippocampal cultures were
20 treated with chloramphenicol to selectively inhibit
21 translation of mitochondrially encoded proteins but not
22 translation of proteins encoded in the nucleus (Ibrahim et
23 al. Journal of Biological Chemistry 251:108-115 1976). After
24 48 hours treatment with chloramphenicol it was found that the
25 level of ND2 in the cultures was reduced by more than 95%

1 whereas there was no significant change in the levels of the
2 nuclear encoded proteins examined (Figure 5A). Figure 5A
3 shows immunoblots of total soluble protein obtained from
4 cultured rat hippocampal neurons treated with 50 µg/ml
5 chloramphenicol for 48 hours and probed with anti-ND2, anti-
6 NR1 and anti-Src as indicated. Importantly, chloramphenicol
7 did not affect the level of Src or of the NMDAR subunit NR1
8 but did suppress the co-immunoprecipitation of Src with the
9 NMDAR complex (Figure 5B), as predicted if ND2 is an adapter
10 protein linking Src to the complex. Figure 5B shows an
11 immunoblot of co-immunoprecipitates obtained from cultured
12 hippocampal neurons, either treated or untreated with 50
13 µg/ml chloramphenicol for 48 hours and probed with anti-NR1
14 or anti-Src.

15 The effect of the 48 hours treatment with
16 chloramphenicol on the ATP levels, mitochondrial membrane
17 potential, viability and general functioning of the
18 hippocampal neurons in culture was examined. It was found
19 that chloramphenicol did not significantly affect the level
20 of ATP levels in the cultures (Figure 5C), consistent with
21 the lack of effect of chloramphenicol treatment for up to 55
22 hours on ATP levels in other cell types in culture
23 (Ramachandran et al. Proceedings of the National Academy of
24 Science USA 99:6643-6648 2002). Figure 5C shows summary

1 histograms (left panel) of ATP level or mitochondrial
2 membrane potential ($\Delta\psi$ M), as assessed by TMRM fluorescence
3 dequenching (right panel), in cultured hippocampal neurons
4 either untreated or treated 50 μ g/ml chloramphenicol for 48
5 hours. To examine the effect of chloramphenicol on
6 mitochondrial membrane potential ($\Delta\psi$ M) in individual neurons,
7 the dequenching of the potentiometric fluorescent cationic
8 dye tetramethylrhodamine methyl ester (TMRM) by the
9 mitochondrial uncoupler carbonyl cyanide p-
10 trifluoromethoxyphenylhydrazone (FCCP) was monitored (Reers
11 et al. Biochemistry 30:4480-4486 1991). The dequenching
12 response evoked by bath-applied FCCP (2 μ M) in neurons from
13 chloramphenicol-treated or control cultures was assessed. It
14 was found that the dequenching response of chloramphenicol-
15 treated neurons was not different from that of untreated
16 neurons (Figure 5C), indicating that $\Delta\psi$ M was not affected by
17 chloramphenicol. Moreover, it was found that neurons treated
18 with chloramphenicol were not distinguishable from untreated
19 neurons in terms of cell number, gross morphology, resting
20 membrane potential, resting intracellular calcium
21 concentration, action potential amplitude, or mEPSC frequency
22 (data not illustrated). Thus, from these data together it
23 was concluded that treatment with chloramphenicol for 48
24 hours did not detectably compromise the functioning of the

1 neurons. Nevertheless, it was noted that the intracellular
2 solution used for all whole-cell recordings contained 2 mM
3 Mg-ATP, so that the level of intracellular ATP was equal in
4 all cells throughout the experiments.

5 In neurons treated with chloramphenicol for 48 hours it
6 was found that the NMDAR component of the mEPSCs was not
7 affected by administering either the EPQ(pY)EEIPIA (SEQ ID
8 NO:5) peptide or the Src40-58 peptide (Figures 5D-F). In
9 contrast, in control experiments administering
10 EPQ(pY)EEIPIA(SEQ ID NO:5) increased the NMDAR component of
11 mEPSCs by $172 \pm 28\%$ and application of Src40-58 decreased the
12 NMDAR component to $56 \pm 4\%$ (Figures 5D-F). Chloramphenicol was
13 present during the recording periods of the control
14 experiments and therefore the loss of effect of the
15 EPQ(pY)EEIPIA(SEQ ID NO:5) and Src40-58 peptides cannot be
16 attributed to an acute effect of chloramphenicol. Figure 5D
17 shows that the upregulation of NMDAR activity in the presence
18 of the Src activator peptide EPQ(pY)EEIPIA(SEQ ID NO:5),
19 labeled as (pY)EEI (amino acid residues 4-7 of SEQ ID NO:5),
20 is prevented in neurons treated with chloramphenicol for 48
21 hours. Figure 5E shows that the reduction of NMDA activity in
22 the presence of the Src40-58 peptide is also prevented in
23 neurons treated with chloramphenicol for 48 hours. Composite
24 traces are shown in black, the NMDAR component in dark grey,

1 and the AMPAR component in light grey. Scale bars are
2 50ms/10pA. Figure 5F shows a summary histogram of
3 electrophysiology data. NMDA component data were calculated
4 as Q_{20}/Q_2 , and AMPA component data were calculated as A_{20}/A_2 .
5 A 48 hour chloramphenicol treatment prevents the modulation
6 of NMDAR function by the Src activator peptide (SEQ ID NO:5)
7 and Src40-58 peptides, while neither of these reagents
8 affected the AMPA receptor component of the MEPSCs under the
9 recording conditions used. An * indicates a significant
10 difference, Student's t-test, $p < 0.05$. Taking our results
11 together, it is concluded that Src-dependent regulation of
12 the activity of NMDARs depends on expression of ND2 through
13 its anchoring of Src to the NMDAR complex.

14
15 EXAMPLE 6

16
17
18 Src40-49 interacts directly with ND2

19
20 To detect the binding of ND2.1-GST with Src peptides,
21 the ND2.1-GST fusion protein was purified on glutathione
22 SEPHAROSE. Src40-58, Src40-49, Src49-58, and scrambled
23 Src40-58 peptides (30 mg/ml; synthesized by HSC Peptide
24 Synthesis Facility; all four peptides are schematically
25 depicted in Figure 6A) were biotinylated by incubating with
26 Sulfo-NHS-Biotin (Pierce Chemical Co., Rockford, IL) for 30

1 minutes at room temperature. The biotinylation reaction was
2 then quenched by the addition of Tris-HCl (pH 8.0) to a final
3 concentration of 20 mM. Biotinylated peptides were incubated
4 with ND2.1-GST on beads for 1 hour at 4°C. The beads were
5 washed three times with PBS/0.1% Triton X-100, then
6 resuspended in PBS+SDS-PAGE sample buffer. After brief
7 centrifugation, samples were resolved by SDS-PAGE,
8 transferred to nitrocellulose membranes, and probed using a
9 streptavidin-HRP conjugate (Sigma, St. Louis, MO). Bound
10 probe was then detected on film using an ECL kit (Amersham
11 Pharmacia Biotech, Baie d'Urfé, Québec). Figure 6B shows the
12 blot of the ND2.1-GST fusion protein which was probed with
13 biotinylated Src peptides followed by streptavidin-HRP
14 conjugate.

15 Src40-58, Src40-49, Src49-58, scrambled Src40-58,
16 TAT-Src40-49, and scrambled TAT-Src40-49 peptides were
17 biotinylated by incubating with Sulfo-NHS-Biotin (Pierce
18 Chemical Co., Rockford, IL) for 30 minutes at room
19 temperature. The biotinylation reaction was then quenched by
20 the addition of Tris-HCl (pH 8.0) to a final concentration of
21 20 mM. Purified recombinant fusion proteins (~20 µg each)
22 were dotted onto nitrocellulose and dried overnight.
23 Membranes were blocked with 5% BSA in PBS (pH 7.5) for 1
24 hour, after which biotinylated peptides (30 µg/ml) diluted

1 1:1000 in fresh 5% BSA in PBS were added. The membranes were
2 incubated with the peptides for 1 hour, washed, and probed
3 with streptavidin-HRP conjugate. Bound probe was then
4 detected on film using an ECL kit. Figure 6C shows the dot
5 blots of ND2.1-GST fusion proteins probed with biotinylated
6 Src peptides followed by streptavidin-HRP conjugate.

7
8 EXAMPLE 7
9

10 TAT-Src40-49 (TSUDAPI-1) reduces pain behavior
11

12 Male Sprague-Dawley rats 150-200 g were used for all
13 experiments. Rats were housed in pairs, maintained on a
14 12/12 hour light/dark cycle, and allowed free access to food
15 and water. All experiments were conducted during 10 am and
16 5pm.

17 Peptide Src40-49Tat (TSUDAPI-1; SEQ ID NO:2) or Tat
18 alone (amino acid residues 1-11 of SEQ ID NO:2) was dissolved
19 in sterilized saline. Peptide or saline was injected
20 intravenously at a volume 1ml/Kg into rat's tail 45 minutes
21 before behavioral testing. Injections were done under brief
22 halothane anesthesia and rats were returned to the cages
23 after injections.

24 The formalin test was performed as previously described
25 (Liu et al. European Journal of Pharmacology 408(2):143-152
26 2000). Rats were placed in a plexiglass observation chamber

1 for an initial 20 minutes to allow acclimatization to the
2 testing environment. Formalin 2.5% was injected
3 subcutaneously in a volume of 50 μ l into the plantar aspect
4 of the hind paw. Following injections, rats were returned to
5 the observation chamber and monitored for flinching behaviors
6 (lifting, shaking and overt flinching with a ripple over the
7 haunch) and biting/licking time. Two rats in adjacent
8 chambers were observed at one time, with observations
9 occurring in alternate 2 minute bins. Recorded episodes were
10 not corrected, thus values represent about half of the total
11 behaviors expressed.

12 Figures 7A-D show the effect of Src40-49Tat (0.1 μ mol) on
13 2.5% formalin induced flinching or biting/licking behaviors.
14 Peptides or saline controls were injected 45 minutes before
15 behavioral testing. Figure 7B shows measurement of flinching
16 behaviors observed within an hour. Figure 7A shows the
17 cumulative flinches in different phases observed within the
18 hour. P1 represents a time period of 0-8 minutes; P2A
19 represents a time period of 12-28 minutes and P2B represents
20 a time period of 32-60 minutes. Values depict means (n=7,
21 Src40-49Tat; n=20, saline). $P < 0.05$, $P < 0.01$ with student t
22 test compared to saline control. Figure 7D shows measurement
23 of the time of each biting/licking behavior observed within
24 an hour. Figure 7C shows the cumulative biting/licking

1 behaviors in different phases observed within the hour. P1
2 represents a time period of 0-8 minutes; P2A represents a
3 time period of 12-28 minutes and P2B represents a time period
4 of 32-60 minutes. Values depict means (n=7, Src40-49Tat;
5 n=20, saline). $P < 0.05$, $P < 0.01$ with student t test compared to
6 saline control.

7 Figures 8A-D show the effect of HIV-Tat (1pmol/g) on
8 2.5% formalin induced flinching or biting/licking behaviors.
9 Peptides or saline controls were injected 45 minutes before
10 behavioral testing. Figure 8B shows measurement of flinching
11 behaviors observed within an hour. Figure 8A shows the
12 cumulative flinches in different phases observed within the
13 hour. P1 represents a time period of 0-8 minutes; P2A
14 represents a time period of 12-28 minutes and P2B represents
15 a time period of 32-60 minutes. Values depict means (n=7,
16 HIV-Tat; n=20, saline). $P < 0.05$, $P < 0.01$ with student t test
17 compared to saline control. Figure 8D shows measurement of
18 the time of each biting/licking behavior observed within an
19 hour. Figure 8C shows the cumulative biting/licking
20 behaviors in different phases observed within the hour. P1
21 represents a time period of 0-8 minutes; P2A represents a
22 time period of 12-28 minutes and P2B represents a time period
23 of 32-60 minutes. Values depict means (n=7, HIV-Tat; n=20,
24 saline). $P < 0.05$, $P < 0.01$ with student t test compared to

1 saline control. As compared to HIV-Tat alone and the saline
2 control, the Src40-49Tat peptide is shown to reduce pain
3 behaviors over a time period of an hour.
4
5

6 EXAMPLE 8

7 ND2-Src interaction in multiple tissues

8
9
10 Total soluble protein was prepared from pre-weighed rat
11 tissues by homogenization at 4°C in 0.25 M sucrose/10mM
12 HEPES-NaOH, 1mM EDTA, pH 7.4 with 2µg/ml each of aprotinin,
13 pepstatin A, and leupeptin. Following brief configuration of
14 the samples at 4 000g, NP-40 was added to 1% (vol/vol) to the
15 cleared supernatants. After incubation for 10 minutes, the
16 protein concentration of the samples was determined by
17 detergent compatible protein assay (BioRad Laboratories,
18 Mississauga, Ontario) and equilibrated. The solubilized
19 proteins were centrifuged briefly at 14 000g to remove
20 insoluble material and then incubated with 5µg of either
21 anti-ND2 (rabbit polyclonal from Dr. R.F. Doolittle, UCSD,
22 CA; described in Mariottini et al. PNAS USA 83:1563-1567
23 1986), anti-Src (mouse monoclonal clone 327 from J. Bolen,
24 DNAX, Palo Alto, CA) or control, non-specific rabbit or mouse
25 IgG (Sigma) overnight at 4°C. Immune complexes were isolated
26 by the addition of 10µl of protein G-SEPHAROSE beads followed
27 by incubation for 2 hours at 4°C. Immunoprecipitates were
28 then washed three times with RIPA buffer, re-suspended in

1 RIPA buffer + SDS-PAGE sample buffer and boiled for 5
2 minutes. The samples were resolved by SDS-PAGE, transferred
3 to nitrocellulose membranes and analyzed by immunoblotting
4 with anti-ND2, anti-Src or anti-Fyn (mouse monoclonal clone
5 25, Pharmingen, Mississauga, Ontario). Bound antibody was
6 then detected on film using appropriate secondary
7 antibody/HRP conjugates and an ECL kit (Amersham Pharmacia
8 Biotech). For control immunoprecipitations under denaturing
9 conditions, SDS was added to the initial protein samples to a
10 final concentration of 0.4% and the samples were boiled for 5
11 minutes and rapidly cooled to 4°C prior to the addition of
12 the antibodies used for immunoprecipitation. In addition,
13 pre-adsorption of the anti-ND2 antibody with antigenic
14 peptide prevented antibody signal detection on immunoblots.

15 Non-receptor tyrosine kinase Src and ND2 are both
16 expressed in cells of multiple, diverse tissues.
17 Illustrative, albeit non-limiting, examples are peripheral
18 nervous system tissue, central nervous system tissue, heart,
19 intestine, kidney, liver, lung, pancreas, skeletal muscle,
20 spleen, testis, bone, skin and brain. The data presented in
21 Figures 10A-B shows that ND2 and Src interact in multiple,
22 diverse tissues. Immunoblots of co-immunoprecipitates from
23 various tissues (Figure 10A) and tissue homogenates (Figure
24 10B) probed with anti-ND2, anti-Src, or anti-Fyn as

1 indicated. Tissues: B-brain; H-heart; I-intestine; K-kidney;
2 Liv-liver; Lu-lung; P-pancreas; Sk-skeletal muscle; Sp-spleen
3 and T-testis. Non-specific IgG applied to liver homogenate
4 was used as a negative control for co-immunoprecipitation.
5 Immunoblotting of Fyn protein from brain was used as a
6 positive control for the anti-Fyn antibody. In these
7 experiments the cell lysates were prepared using non-
8 denaturing conditions, but when denaturing conditions were
9 used to prepare the proteins, no co-immunoprecipitation of
10 Src by anti-ND2 or of anti-Src was found (data not
11 illustrated).

12

13 IN SUMMARY

14 The main criteria for identifying ND2 as the protein
15 mediating the interaction between NMDARs and the unique
16 domain of Src, as inferred from previous work (Ali et al.
17 Current Opinion in Neurobiology 11:336-342 2001; Yu et al.
18 Science 275:674-678 1997) are as follows: ND2 must bind
19 directly to the unique domain of Src through amino acids 40-
20 58 (specifically 40-49; SEQ ID NO:1); this binding must be
21 prevented by the Src40-58 (specifically 40-49) peptide; ND2
22 must be present at excitatory synapses and must be a
23 component of the NMDAR complex; and lack of ND2 must prevent
24 the upregulation of NMDAR activity by endogenous Src.

1 ND2 was first considered as a potential Src unique
2 domain-binding protein when overlapping clones of ND2 in two
3 separate yeast two-hybrid experiments were isolated.
4 Subsequently, the direct interaction of the Src unique domain
5 and ND2 was confirmed through in vitro binding assays using
6 recombinant proteins. Through these experiments the ND2.1
7 region was identified as necessary and sufficient for
8 interacting with the Src unique domain. ND2.1 bound directly
9 to the Src40-58 (specifically 40-49)peptide and the in vitro
10 binding of the Src unique domain to ND2.1 was prevented by
11 Src40-58 (specifically 40-49). Src and ND2 co-
12 immunoprecipitated with each other in brain homogenates and
13 PSD protein preparations. The co-immunoprecipitation was
14 prevented by Src40-58(specifically 40-49), implying that the
15 Src-ND2 interaction identified in vitro may occur in vivo.
16 In addition to finding ND2 in PSD protein preparations, ND2-
17 immunoreactivity was found by immunogold electron microscopy
18 in PSDs in the CA1 hippocampus. Moreover, co-
19 immunoprecipitation experiments indicated that ND2 is a
20 component of the NMDAR complex and that the Src-ND2
21 interaction is required for the association of Src, but not
22 ND2, with NMDARs. It was found that depleting ND2 suppresses
23 Src association with the NMDAR complex and prevents the
24 upregulation of NMDAR function by activating endogenous Src

1 at excitatory synapses. Src40-49 (SUDAPI-1;SEQ ID NO:1) was
2 identified as the specific peptide that interacts with ND2 as
3 Src50-58 alone did not interact with ND2. Finally, it was
4 found that TAT-Src40-49 (TSUDAPI-1;SEQ ID NO:2) as
5 administered to rats reduced pain behavior in the formalin
6 test. These multiple, converging lines of evidence lead to
7 the conclusion that ND2 is the protein mediating the
8 interaction between NMDARs and the unique domain of Src.

9 ND2 is mitochondrially encoded and translated, and yet
10 it is found within the PSDs of glutamatergic synapses in the
11 brain. The other mitochondrial proteins examined, ND4 and
12 Cyto 1, were not detected in the PSD fraction implying that
13 this fraction is not contaminated non-specifically by
14 mitochondrial proteins. Further, ND2-immunoreactivity by
15 immunogold electron microscopy was found within structurally-
16 identified PSDs in dendritic spines of CA1 neurons. In this
17 preparation, proteins are immobilized by tissue fixation
18 precluding the possibility that ND2 could have relocated from
19 the mitochondria to the PSD during processing. Moreover,
20 because dendritic spines are devoid of mitochondria (Shepherd
21 et al. Journal of Neuroscience 18(20):8300-8310 1998) the ND2
22 immunoreactivity cannot be accounted for by mitochondria
23 abutting the PSD. Taken together these findings indicate
24 that ND2, but not the entire Complex I, is normally present

1 within the PSD. The PSD contains many enzymes that may be
2 involved in regulating synaptic functioning (P. Siekevitz
3 Proceedings of the National Academy of Science USA 82:3494-
4 3498 1985) including glycolytic enzymes capable of
5 generating ATP (Wu et al. Proceedings of the National Academy
6 of Science USA 94:13273-13278 1997). However, without other
7 components of Complex I it is unlikely that ND2 functions
8 catalytically within the PSD.

9 Thus, in addition to its localization in mitochondria
10 and function as a component of Complex I, the present results
11 indicate that ND2 has a second location and function in
12 outside the mitochondria. Mitochondria are intimately linked
13 to overall cellular functioning through generation of ATP by
14 oxidative phosphorylation. Mitochondria are also known to be
15 key for sequestration of intracellular calcium (D.D. Friel
16 Cell Calcium 28:307-316 2000; R. Rizzuto Current Opinion in
17 Neurobiology 11:306-311) and to participate in programmed
18 cell death (Gorman et al. Developmental Neuroscience 22:348-
19 358 2000; M.P. Mattson National Review of Molecular and
20 Cellular Biology 1:120-129 2000). Some mitochondrial proteins
21 are known to be present at extra-mitochondrial sites (Soltys
22 et al. Trends in Biochemical Science 24:174-177 1999; Soltys
23 et al. International Review of Cytology 194:133-196 1999).
24 But, the experiments described herein indicate a new type of

function for a mitochondrial protein outside this organelle, that is ND2 acts as an adapter protein that anchors Src within the NMDAR complex, where it thereby allows Src to upregulate NMDAR activity.

Upregulating the activity of NMDARs is a major function of Src in neurons in the adult CNS (Lu et al. Science 279:1363-1368 1998; Pelkey et al. Neuron 34:127-138 2002; Huang et al. Neuron 29:485-496 2001) and this mediates the induction of long-term potentiation (LTP) of excitatory synaptic transmission in CA1 neurons in the hippocampus (Ali et al. Current Opinion in Neurobiology 11:336-342 2001). The findings described herein imply that the ND2-Src interaction is essential for LTP induction as LTP in CA1 neurons is prevented by Src40-58 and by anti-Src1, an antibody that recognizes this amino acid sequence within the Src unique domain and which prevents the Src unique domain interaction with ND2.1 in vitro (J.R.G., M.W.S. unpublished observations). LTP at Schaffer collateral-CA1 synapses is the prototypic example of NMDAR-dependent enhancement of excitatory synaptic transmission, which is observed at numerous types of glutamatergic synapses throughout the CNS (Malenka et al. Science 285:1870-1874 1999). In addition, Src has been implicated in NMDAR-dependent seizures (Sanna et al. Proceedings of the National Academy of Science 97:8653-

1 8657 2000), chronic pain (Guo et al. Journal of Neuroscience
2 22:6208-6217 2002) and neurotoxicity (Pei et al. Journal of
3 Cerebral Blood Flow Metabolism 21:955-963 2001). Thus, the
4 discovery of the Src-ND2 interaction at NMDARs, which is
5 disclosed herein, defines a protein-protein interaction of
6 general relevance to regulation of neuronal function,
7 synaptic plasticity, and pathophysiology in the CNS.

8 Additionally, by showing an extramitochondrial action
9 for a protein encoded in the mitochondrial genome a
10 previously unsuspected means by which mitochondria regulate
11 cellular function has been identified. Because ND2 and Src
12 are broadly expressed, the interaction of ND2 with the Src
13 unique domain may be of general relevance for control of Src
14 signaling (Example 8 and Figures 10A-B).

15 All patents and publications mentioned in this
16 specification are indicative of the levels of those skilled
17 in the art to which the invention pertains. All patents and
18 publications are herein incorporated by reference to the same
19 extent as if each individual publication was specifically and
20 individually indicated to be incorporated by reference.

21 It is to be understood that while a certain form of the
22 invention is illustrated, it is not to be limited to the
23 specific form or arrangement herein described and shown. It
24 will be apparent to those skilled in the art that various

1 changes may be made without departing from the scope of the
2 invention and the invention is not to be considered limited
3 to what is shown and described in the specification. One
4 skilled in the art will readily appreciate that the present
5 invention is well adapted to carry out the objectives and
6 obtain the ends and advantages mentioned, as well as those
7 inherent therein. The oligonucleotides, peptides,
8 polypeptides, biologically related compounds, methods,
9 procedures and techniques described herein are presently
10 representative of the preferred embodiments, are intended to
11 be exemplary and are not intended as limitations on the
12 scope. Changes therein and other uses will occur to those
13 skilled in the art which are encompassed within the spirit of
14 the invention and are defined by the scope of the appended
15 claims. Although the invention has been described in
16 connection with specific preferred embodiments, it should be
17 understood that the invention as claimed should not be unduly
18 limited to such specific embodiments. Indeed, various
19 modifications of the described modes for carrying out the
20 invention which are obvious to those skilled in the art are
21 intended to be within the scope of the following claims.